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Probability Distribution of Drug-Resistant Mutants in Unselected Populations of *Mycobacterium tuberculosis*

HUGO L. DAVID

Mycobacteriology Unit, Center for Disease Control, Atlanta, Georgia 30333

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The fluctuation test shows that *Mycobacterium tuberculosis* mutates to resistance to isoniazid, streptomycin, ethambutol and rifampin spontaneously and at random. The average mutation rates for the drugs, in the same order, were calculated to be 2.56×10^{-8}, 2.95×10^{-8}, 10^{-7}, and 2.25×10^{-10} mutation per bacterium per generation. The relatively high mutation rate to ethambutol resistance and the low mutation rate to rifampin resistance were confirmed by analyzing the increase in the proportion of mutants with time in a growing population of the tubercle bacilli. The highest proportions of mutants to be expected in unselected populations of the tubercle bacilli were calculated from the results of fluctuation tests.

By definition, a drug-resistant population of bacteria is composed of cells able to survive exposure to a certain concentration of the drug. Yet, in pulmonary tuberculosis, failure of chemotherapy may be apparent even though only a certain proportion of the cells in the bacterial population are resistant to the corresponding drug (14). Since mutations occur spontaneously and at random (4, 10, 11), the highest proportion of mutants to be expected in unselected populations of the tubercle bacilli and the mutation rates of this organism to the various antituberculous drugs are of importance, both in the performance and the interpretation of drug-sensitivity tests in tuberculosis (3, 12). Therefore, it seemed worthwhile to apply the fluctuation test designed by Luria and Delbrück (11) to *Mycobacterium tuberculosis*. The drugs investigated were isoniazid (INH), streptomycin (STM), ethambutol (Eta), and rifampin (Rif).

MATERIALS AND METHODS

Strain and conditions of growth. The strain H37Rv of *M. tuberculosis* was used in all experiments. The cells were grown in Middlebrook and Cohn 7H9 medium (Difco) containing 0.05% Tween 80 and the ADC enrichment. Large cultures were grown in 200-ml volumes of 7H9 medium dispensed in 500-ml nephelometric flasks; small cultures were grown in 2.0- or 5.0-ml volumes of the same medium dispensed in screw-capped test tubes (16 by 125 mm). All stationary cultures were incubated at 35 to 36 C in an atmosphere of 5 to 10% carbon dioxide. Drug-resistant mutants were identified by inoculating samples of the cultures in liquid medium onto the surface of Middlebrook 7H10 agar medium (Difco) containing appropriate concentrations of the drugs, dispensed in disposable quadrant plates. The inoculated drug-plates were incubated for 4 weeks before reading.

Fluctuation test. At approximately the middle of the exponential phase of growth, samples of the large cultures were diluted so that 1.0 ml of the dilutions contained about 10,000 cells. Samples (0.1 ml) of the dilution (about 1,000 cells) were inoculated in 2.0 or 5.0 ml of 7H9 medium. After 3 weeks of incubation, 0.1 ml of each of the small, identical cultures was inoculated on 7H10 medium containing drugs.

The number of cells inoculated in all of the small, identical cultures and the amount of growth in the 2.0-ml small cultures at the time of testing were estimated by diluting and plating the cells on drug-free 7H10 agar medium dispensed in disposable petri dishes (100 by 15 mm). The amount of growth at the time of testing in the 5.0-ml cultures was estimated by converting the absorbancy at 650 nm, read in a Coleman Jr. Spectrophotometer, to the total number of viable cells.

The mutation-rates were calculated by the Luria and Delbrück formula (11) applicable to a fluctuation test performed as described above:

$$r = a.Nt.ln\ (Nt.C.a) \qquad (1)$$

In this formula, a = mutation rate, r = average number of mutants, Nt = total number of cells at the time of testing, and C = number of cultures tested.

Estimation of mutation rates by Stocker's (15) procedure is as follows. The number of cells and the number of Eta- and Rif-resistant mutants in an actively growing culture of the tubercle bacilli at two

different times were estimated by diluting and plating the culture on drug-free and drug-containing 7H10 agar medium. The number of generations that elapsed during the experiment was calculated by the formula

$$n = \frac{\log Nt - \log No}{0.301} \quad (2)$$

in which n = number of generations, No = number of cells at time 0, and Nt = number of cells at time t. The mutation rates were calculated by the formula

$$a = 2.\, ln\, 2. \left(\frac{Mt}{Nt} - \frac{Mo}{No}\right) \Big/ n \quad (3)$$

in which Mo = number of mutants at time 0 and Mt = number of mutants at time t; a, Mt, Nt and n have the same meaning as in equations 1 and 2.

Drugs and drug concentrations in 7H10 medium. INH, STM, Eta and Rif were gifts of, respectively, Panray Division, Ormont Drug & Chemical Co., Englewood, N.J.; Merck & Company, Inc., Rahway, N.J.; Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.; and Pitman-Moore Division, Dow Chemical Co., Indianapolis, Ind. The drug concentrations used in these experiments were: INH, 0.2 and 1.0 μg/ml; STM, 2.0 μg/ml; Eta, 5.0 μg/ml; and Rif, 1.0 μg/ml. These are the concentrations used in the drug-sensitivity method in this laboratory (18).

Definitions. In this report an unselected population refers to a population which was not subject to a direct (in consequence of a previous exposure to the drug) or indirect (or sib-selection, 4) selection. Except when otherwise stated, the expression "selected population" will apply to a population subject to direct selection of mutants.

When applied to populations, the expression drug-resistant is used to indicate a bacterial population in which the proportion of the cells capable of surviving exposure to the indicated concentration of the drug approaches 1.0 (100%). Populations containing variable proportions of drug-resistant mutants are collectively referred to as selected populations or are characterized by indicating the percentage of mutants.

RESULTS

The results obtained in fluctuation tests applied to the tubercle bacilli are shown in Table 1. A wide fluctuation in the frequency of mutants between identical cultures was observed in the experiments performed with INH, STM, and Eta. Rif-resistant mutants appeared to occur rarely. The highest proportions of mutants observed were 3.5×10^{-6} and 3.1×10^{-6} for, respectively, INH, 0.2 and 1.0 μg/ml; and 3.8×10^{-6}, 3.1×10^{-8}, and 0.5×10^{-4} for, respectively, STM, Rif, and Eta. The mutation rates for the drugs, in the same order, were calculated to be 2.56×10^{-8}, 2.95×10^{-8}, 2.25×10^{-10}, and 1.0×10^{-7} mutations per bacterium per generation.

The low mutation rate to resistance to Rif was confirmed by performing a fluctuation analysis with a large inoculum (1.5×10^6 cells). The results of the experiment are depicted in Table 2. The highest proportion of mutants observed was 3×10^{-9}, and the mutation rate was calculated to be 7.53×10^{-10} mutations per bacterium per generation.

The increase in the proportion of mutants resistant to Rif and Eta in an actively growing population of the tubercle bacilli is shown in Table 3. The mutation rates for these drugs calculated by equation (3) were, respectively, 3.32×10^{-9} and 6.4×10^{-7} mutations per bacterium per generation.

DISCUSSION

The purpose of these investigations was not to verify whether drug resistance in the tubercle bacilli was due to the spontaneous and random occurrence of mutations. However, since evidence indicating that drug-resistant mutants of the tubercle bacilli arise at random in growing populations is not available, it is worthwhile to emphasize that these results of the fluctuation analysis conclusively confirm the spontaneous and random occurrence of drug resistance in this organism. The presence of spontaneous STM-resistant mutants in populations of *M. tuberculosis* was demonstrated previously by Schaefer (*personal communication to* Middlebrook and Russel, 13) who applied the Lederberg and Lederberg replica-plating method (10).

The proportion of drug-resistant mutants in unselected populations of the tubercle bacilli has been established by finding the proportion of mutants in laboratory strains or in strains isolated from the pathological discharges of untreated patients. Various investigators have tested populations from large cultures. Although the size of the mutant clones in replicates of each culture was approximately the same (3, 5, 9, 19), the sizes of the clones between strains varied considerably (1–3, 5, 8, 9, 13, 19). The reason for the wide fluctuation in the proportion of mutants between strains is clearly indicated by the results of the fluctuation analysis: the size of the mutant clone depends upon the time when the first mutation occurred and, therefore, the proportion of mutants in a growing culture should increase with time (6, 11, 15). Since each cell in any of the strains tested has a small but fixed chance to mutate (11), as much variation in the proportion of mutants within each strain can be observed as between several strains tested at a given time. Therefore, a frequency distribution of unselected strains as a function of the proportion of mutants found at the time of testing does not describe the

TABLE 1. *Fluctuation test as applied to Mycobacterium tuberculosis strain H37Rv*

Determination	Isoniazid				Streptomycin		Rifampin		Ethambutol	
	0.2 μg/ml		1.0 μg/ml		2.0 μg/ml		1.0 μg/ml		5.0 μg/ml	
No. of cultures	20	39	20	42	20	45	20	46	20	46
Vol. of culture	2.0	5.0	2.0	5.0	2.0	5.0	2.0	5.0	2.0	5.0
Vol. of sample	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Resistant bacteria:										
0	1	2	3	4	9	7	19	42	12	15
1	1	0	5	1	8	0	1	3	1	1
2	2	0	3	1	2	1	0	1	1	0
3	4	0	1	0	0	0	0	0	0	2
4	1	0	0	0	1	0	0	0	0	0
5	0	0	2	0	0	0	0	0	0	0
6–10	5	2	1	2	0	0	0	0	0	2
11–20	2	10	3	5	0	4	0	0	1	1
21–50	3	16	0	20	0	18	0	0	2	11
51–100	0	9	1	7	0	11	0	0	1	7
101–200	0	0	0	2	0	2	0	0	0	0
201–500	1	0	1	0	0	2	0	0	1	5
501–1000	0	0	0	0	0	0	0	0	1	1
1,000+	0	0	0	0	0	0	0	0	0	1
Average mutants per ml	190	907	170	862	80	1305	0.5	3.1	843	4040
Bacteria/ml	1.62×10^9	3.15×10^9	1.62×10^9	3.15×10^9	1.62×10^9	3.15×10^9	1.62×10^9	3.15×10^9	1.62×10^9	3.15×10^9
Highest proportion of mutants	3.50×10^{-6}	1.5×10^{-6}	3.1×10^{-6}	2.0×10^{-6}	0.5×10^{-7}	3.8×10^{-6}	1.2×10^{-8}	3.1×10^{-8}	1.2×10^{-5}	0.5×10^{-4}
Mutation rate	1.84×10^{-8}	3.5×10^{-8}	1.7×10^{8}	3.2×10^{-8}	0.9×10^{-8}	5.0×10^{-8}	1.8×10^{-10}	2.7×10^{-10}	0.7×10^{-7}	1.3×10^{-7}
Average mutation rate		2.56×10^{-8}			2.95×10^{-8}		2.25×10^{-10}		1.0×10^{-7}	

events that take place in each population. Unselected populations can be characterized by using two parameters obtained in the fluctuation analysis: the highest proportion of mutants that can be expected and the chance of a mutation occurring per bacterium per generation or generation time (6).

The highest proportion of mutants that can be expected in unselected populations of the tubercle bacilli was observed to be 3.5×10^{-6} and 3.1×10^{-6} to 0.2 and 1.0 μg of INH per ml; and 3.8×10^{-6}, 3.1×10^{-8}, and 0.5×10^{-4} to, respectively, 2.0 μg of STM per ml, 1.0 μg of Rif per ml, and 5.0 μg of Eta per ml. The mutation rates to resistance to the drugs were found to be, in the same order, 2.56×10^{-8}, 2.95×10^{-8}, 2.25×10^{-10}, and 1.0×10^{-7} mutations per bacterium per generation. The mutation rates of *M. ranae* to resistance to 25 μg of STM per ml and 500 μg of INH per ml were previously calculated to be 10^{-9} and 10^{-6} to 3×10^{-6} per bacterium per generation (7, 16).

The highest proportion of mutants that can be expected in unselected populations of the tubercle bacilli is a useful figure when one's purpose is to establish whether a population is subject to selective pressures. Selected populations, in contrast to unselected populations, can be accurately characterized by finding a proportion of mutants, provided that the initial number of mutants is high enough that the low mutation rate will not significantly change the relative ratio of resistant to sensitive cells. Although bacteriological evidence for selection can be obtained at very low values in the proportion of mutants (4), it is doubtful whether such precision is of practical value.

Evidence indicates that when the proportion of mutants attains a certain level, not necessarily the same for every drug, the strain behaves as resistant for practical purposes (1–3, 13). The experiments we described cannot give information regarding the notion that in tuberculosis practice unresponsiveness to therapy may be observed before the infecting bacterial population is resistant. According to Canetti et al. (1), a strain should be considered resistant if it yields 1% INH-resistant mutants to 0.2 μg/ml, 10% STM-resistant mutants to 4.0 μg/ml, 10% Eta-resistant mutants to 2.0 μg/ml, and 1% Rif-resistant mutants to 40.0 μg/ml.

Our investigations also indicate that there is no reason to hypothesize the occurrence of acquired resistance in the tubercle bacilli. The expressions primary and acquired resistance, used in connection with tuberculosis, are operational and must not be interpreted as having a bacteriological counterpart. If, indeed, primary-resistant cases may respond to therapy with the corresponding drugs in contrast to acquired-resistant cases (17), it seems that, for identical criteria of resistance, conditions may develop in the host during previous chemotherapy that favor the selection of drug-resistant mutants. Whether these conditions can or cannot be circumvented may not have anything to do with the bacteria themselves.

TABLE 2. *Fluctuation analysis of mutation from sensitivity to resistance to rifampin*[a]

Culture no.	No. of resistant cells/0.1 ml	Total no. of cells/0.1 ml	Proportion of mutants
1	4	1.3×10^9	3.07×10^{-9}
2	0	9.2×10^8	0
3	1	1.3×10^9	7.6×10^{-9}
4	0	2.5×10^9	0
5	1	1.3×10^9	7.6×10^{-9}
6	0	1.6×10^9	0
7	0	1.3×10^9	0
8	0	2.5×10^9	0
9	0	2.5×10^9	0
10	0	2.0×10^9	0
Average		1.6×10^9	—

[a] Volume of culture, 5.0 ml; inoculum size, 1.5×10^6 cells.

TABLE 3. *Increase in the proportion of mutants as a function of the number of generations elapsed in growing populations of the tubercle bacilli*

Drug	No. of cells at time (days)		No. of mutants at time (days)		No. of generations (n)[a]	Mutation rate (a)[b]
	1 (*No*)	11 (*Nt*)	1 (*Mo*)	11 (*Mt*)		
Rifampin	3×10^6	8.3×10^9	0	20	11.4	3.32×10^{-9}
Ethambutol	3×10^6	8.3×10^9	10	72,000	11.4	6.4×10^{-7}

[a] $n = (\log Nt - \log No)/0.301$.
[b] $a = 2.ln\ 2.\ [(Mt/Nt) - (Mo/No)]/n$.

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